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EVALUATION OF ANTIOXIDANT POTENTIALS OF LEAF AQUEOUS AND METHANOLIC EXTRACTS OF *CALOPHYLLUM INOPHYLLUM* IN RELATION TO TOTAL PHENOL AND FLAVONOID CONTENTS

SUMANA DUTTA AND SANJIB RAY*

Molecular Biology and Genetics Unit, Department of Zoology, The University of Burdwan, Golapbag, Burdwan-713104, West Bengal, India.

ABSTRACT

The leaves, barks, flowers, fruits and seeds of *Calophyllum inophyllum* are widely used in traditional practices. Here in the present study, we aimed to evaluate *in vitro* antioxidant potential of leaf aqueous and methanolic extracts of *C. inophyllum* in relation to total phenol and flavonoid contents. DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging and ferric (Fe^{+3}) reducing power was analyzed for antioxidant activity. The total phenol content was estimated using Folin-Ciocalteu reagent and flavonoid content was estimated spectrophotometrically ($AlCl_3$) and calculated as tannic acid and quercetin equivalent respectively. DPPH free radical scavenging and Ferric (Fe^{+3}) reducing the power of the leaf aqueous and methanolic extracts were increased with the increasing extract concentrations. Leaf methanolic extract of *C. inophyllum* (LMECI) showed more free radical scavenging activity and reducing power than the leaf aqueous extract of *C. inophyllum* (LAECI). Total phenol contents of aqueous and methanolic extracts were 97± 9.2 and 140.28± 17.1 mg/g respectively. Flavonoid contents of the aqueous and methanolic extracts were 88.94±2.94 and 177.06±5.29 mg/g respectively. The present study exhibited that the leaf methanolic extract of *C. inophyllum* have more free radical scavenging and reducing power than that of the aqueous extract. It may be due to the presence of relatively more total phenol and flavonoid contents in the leaf methanolic extract.

KEY WORDS: **poly phenols, flavonoids, *Calophyllum inophyllum*, antioxidant, reducing power.**

SANJIB RAY
Molecular Biology and Genetics Unit, Department of Zoology, The University of Burdwan, Golapbag, Burdwan-713104, West Bengal, India.

INTRODUCTION

Calophyllum inophyllum (Family: Clusiaceae or Guttiferae, Common name: Punnang in Bengali; Alexandra Laurel in English) is a large evergreen tree with medicinal values. It is widely distributed in the tropics of Asia, Africa, tropical America, Australia, Malay Peninsula, Burma, along the East and West coasts of the Peninsula and Ceylon. In India, it is widely distributed mainly in the coastal regions of Maharashtra, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh, Orissa, the Andamans and Arunachal Pradesh[1]. It is an evergreen, ornamental tree with glossy elliptical leaves opposite in arrangement, flowers are white in color and fruits are purplish black drupe with a single seed. Fruits and flowers of *C. inophyllum* are available all over the year. In traditional practice, its leaves are mainly used to treat different skin problems like skin rash and ulcers, pimples, cuts, wounds and sores. Moreover, the leaf infusion is used to cure bacterial and fungal infection, dysentery and sore eyes[1]. In addition the dried leaves of *C. inophyllum* are used for rheumatism. Various medicinal properties like antimicrobial[1], antioxidant and antidyslipidemic[2], analgesic[1], larvicidal[3], HIV1 integrase, protease and reverse transcriptase inhibitor[4], and anticancer activities[5] have been reported. Oxidative stress is associated with majority of pathological conditions like atherosclerosis, cataracts, rheumatism, AIDS, hyperglycemia, cancer and many other auto-immune and old age diseases[6]. Antioxidants help to check the detrimental effects of free radicals and reactive oxygen species (ROS). They can delay, prevent or inhibit the oxidation of other oxidisable molecules by scavenging free radicals and retreating oxidative stress[7]. There are diverse groups of reducing agents like thiols, poly phenols, vitamins C and E, tri-peptide like glutathione, enzymes such as catalase, peroxidase and superoxide dismutase that act to prevent oxidative damages to DNA, proteins and lipids[8,9].

The medicinal value of a plant depends on phytochemical constituents and secondary metabolites[10]. Recently many plants have been found to possess antioxidant activities that are largely due to phenolic components[11,12]. Poly phenols play an important role in human health because of their diverse pharmacological activities like anti-inflammatory, antiallergic, antimicrobial, antiviral and anticarcinogenic[13]. Moreover, they can prevent oxidative modification of low density lipoprotein and lipid peroxidation by means of their antioxidant activities[14]. Many studies have correlated antioxidant activity with high phenolic contents such as in sage, rosemary, oregano and thyme[15]. Flavonoids function primarily as antioxidants as their hydroxyl groups confer scavenging ability[16] and also have cardio protective role[17]. Poly phenols and flavonoids are the major groups of phytochemicals with antioxidant activity. They effectively diminish the oxidative stress induced tissue damage associated with the chronic diseases [18,19]. The renewed interest in the search for antioxidants is mainly due to the pathological conditions linked to with the oxidative stress and reactive oxygen species. At the present state of knowledge the antioxidant potentials of *C.inophyllum* is not well studied. Therefore, this study aimed to explore in vitro antioxidant potentials of leaf aqueous and methanolic extracts of *C.inophyllum* in relation to their total phenol and flavonoid contents. Here, the leaf aqueous and methanolic extracts of *C.inophyllum* were tested for preliminary phytochemicals, total phenol and flavonoid contents, DPPH free radical scavenging and Fe3+ reducing potentials.

MATERIALS AND METHODS

Chemicals

Quercetin was obtained from SIGMA-ALDRICH, St Louis, MO, USA. Tannic acid powder was obtained from HIMEDIA Laboratories Pvt. Ltd., Mumbai, India. Aluminium chloride was obtained from MERCK Specialities Pvt. Ltd., Mumbai, India. Folin-Ciocalteu and Sodium citrate were obtained from BDH Chemicals Ltd., Poole Dorset, UK. Benzene and Ethyl acetate

were obtained from SRL, Pvt. Ltd., Mumbai, India.

Plant products collection, storage and extract preparation

Fresh leaves of *C. inophyllum* were collected from The Burdwan University campus, West Bengal, India. This plant species was identified by Professor Ambarish Mukherjee,Taxonomist, Department of Botany, The University of Burdwan. The voucher specimen (No.BUTBSD014) is maintained in the Department of Zoology for future reference. Collected leaves were washed in tap water, shade dried, directly crushed into small pieces and followed by pulverizing them using an electric grinder (Philips Mixer Grinder HL1605). Ground leaf powder was stored in air tight container for future use. 50 g dried leaf powder was extracted in 500 ml of distilled water, boiled for 30 min in water bath and it was filtered through filter paper. The leaf aqueous extract of *C.inophyllum* (LAECI) was stored at -20°C for further use. Leaf methanolic extract of *C.inophyllum* (LMECI) was prepared in room temperature soaking the leaf powder in methanol for seven days with intermittent agitation. 10 ml of extract was evaporated to complete dryness in the hot air oven at 60 ºC to determine the extract concentration.

DPPH assay

The antioxidant capacity of leaf aqueous and methanolic extracts of *C. inophyllum* was determined by the DPPH free radical scavenging assay according to a standard method[20,21] with slight modification. DPPH (1,1-diphenyl-2-picrylhydrazyl), a free radical, becomes colorless when it accepts an electron or hydrogen radical. It is widely used for the *in vitro* antioxidant assay. Ascorbic acid (10 mg/ml) was used as standard antioxidant. DPPH solution (0.002%) was prepared in methanol. Ascorbic acid, LAECI and LMECI were prepared in different concentrations (5, 10, 15, 20, 25, 50 and 100 µg/ml) in methanol. 3 ml of DPPH solution was mixed with 1 ml of sample solution and standard solution separately in each test tube. The test tubes were then incubated in the dark for 30 min at room temperature and the optical density was measured at 517 nm using Spectrophotometer (UV-1800 Series, Shimadzu, Japan). Percentage of free radical scavenging activity was calculated by the following equation—

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Reducing Power Assay

Reducing power is an indicator of antioxidant activity. Reducing power of LAECI and LMECI was determined according to the method as described by Oyaizu[22] with little modification. Different concentrations (10, 25, 50, 75, 100 µg/ml) of 0.5 ml of ascorbic acid, LAECI and LMECI were taken in the respective test tubes and then 1 ml of phosphate buffer and 1 ml of $K_3Fe(CN)_6$ (1%) were mixed and the mixtures were incubated at 50º C for 20 min. After that 1ml TCA (10%) was added to each tube and centrifuged at 3000 rpm for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml distilled water and 0.1 ml $FeCl_3$ (0.1%) solution. The absorbance was measured at 700 nm using Spectrophotometer. Higher absorbance indicates higher reducing power and that was compared with standard ascorbic acid. The effective extract concentration providing half maximal absorbance (EC_{50}) was calculated from the standard ascorbic acid graph.

Estimation of total phenol content

The total phenol content was measured by using Folin-Ciocalteu reagent [23]. 10µl of each sample was taken in the respective test tubes and the volume was made up to 1 ml by adding distilled water. Then 0.5 ml of Folin-Ciocalteu (1N) reagent was added and mixed properly. Then 2.5 ml 20% sodium carbonate was added to each sample tube and the test tubes were kept in the dark for 40 min. The optical density was measured at 525 nm using Spectrophotometer. The total phenol content was estimated using the tannic acid, a specific

type of commercial polyphenol, standard curve. Standard tannic acid solution (0.5 mg/ml) was prepared by dissolving 5 mg tannic acid in 10 ml distilled water. From this stock solution different standard concentrations (2.5-25μg/ml) were prepared by serial dilution method.

Estimation of total flavonoid content

The total flavonoid content was estimated by Aluminium chloride colorimetric method[24]. 2 ml of distilled water was added to 1 ml of extract (1 mg/ml). 3 ml of Sodium nitrite (5%, 5 g in 100 ml distilled water) and 0.3 ml of Aluminium chloride (10%, 10 g in 100 ml distilled water) were added to the sample. 2 ml of Sodium hydroxide (1M) was added after 6 min and the volume was made up to 10 ml by adding distilled water. The absorbance of the reaction mixture was recorded at 510 nm with Spectrophotometer. The total flavonoid content of the extract was calculated from the quercetin standard plot.

Phytochemical detection

Tests for alkaloids

Extracts were acidified with a few drops of glacial acetic acid and tested for the presence of alkaloids. A few drops of Mayer's reagent (1.36 g of $HgCl_2$ and 5 g of KI in 100 ml distilled water) were added to 1 ml of acidified extract, formation of white or pale yellow precipitate indicates the presence of alkaloids. A few drops of Wagner's reagent (Solution of iodine in potassium iodide) were added to 1 ml of acidified extract and the appearance of a reddish brown precipitate indicates the presence of alkaloids.

Tests for flavonoids

A few magnesium turnings were added to 1 ml of extract and then drop by drop concentrated HCl was added. The appearance of pink scarlet or green to blue color after a few minutes indicates the presence of flavonoids . Addition of few drops of Sodium hydroxide solution to 1 ml of extract resulted into the formation of an intense yellow color which turned colorless on addition to a few drops of after addition of few diluted HCl, indicating the presence of flavonoids.

Test for anthraquinones

Benzene (2 ml) was mixed properly with 2 ml of extract, filtered and 3.5 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the absence of pink, red or violet color in the lower phase indicates the absence of free anthraquinone.

Tests for terpenoids and steroids

To test terpenoids and steroids the procedure as described by Kantamreddi *et al.*[25] was followed with slight modification. In brief, glacial acetic acid, 1 ml, was mixed with 1 ml of aqueous extract and then 1 ml of concentrated sulphuric acid was added through the wall of the test tube that was kept in ice flakes. The appearances of brown and green color indicate the presence of terpenoids and steroids respectively.

Tests for tannins

Few drops of $FeCl_3$ were added to 2 ml of the extract and the formation of bluish black color indicates the presence of tannins.

Test for phlobatannins

To test phlobatanin the procedure as described by Edeoga *et al.*[26] was followed with slight modification. In brief, the extract was boiled with 1 N HCl and the appearance of red precipitate indicates the presence of phlobatannins.

Test for saponins

Few drops of $NaHCO_3$ were added to 5 ml of the extract, shaken vigorously and left for 3 minutes. Formation of honeycomb like stable froth indicates the presence of saponins.

Tests for glycosides

1 ml aqueous NaOH(1N) solution was added to 1 ml of the extract and the absence of pale yellow precipitate indicates the absence of glycosides.

Test for carbohydrates

5 ml Benedict's solution (100 ml of Benedict's solution contains 17.3 g sodium citrate, 10.0 g sodium carbonate, and 1.73 g cupric sulphate pentahydrate) was added to 0.5 ml of extract and boiled for 5 minutes. Absence of colored

precipitate indicates the absence of carbohydrates.

RESULTS

DPPH assay

Data indicate concentration dependent increased free radical scavenging activity of Ascorbic acid, LMECI and LAECI. The LMECI showed more DPPH free radical scavenging activity than LAECI. The ascorbic acid scavenged 94% free radicals while LAECI and LMECI showed 60 and 88.9% respectively at a concentration 100 µg/ml. The EC_{50} values of the ascorbic acid, LMECI and LAECI were 20, 45 and 80 µg/ml respectively.

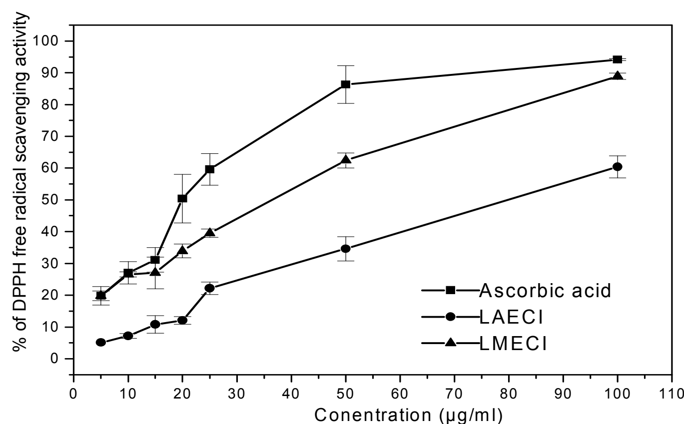

Figure 1
Showing DPPH free radical scavenging activity of Ascorbic acid, LMECI and LAECI. Fe^{3+} reducing property

Data show concentration dependent increased absorbance (OD value) of LAECI and LMECI indicating the increased Fe^{3+} reducing power with the increasing concentrations. The LMECI showed more reducing activity than the LAECI (Fig 2).

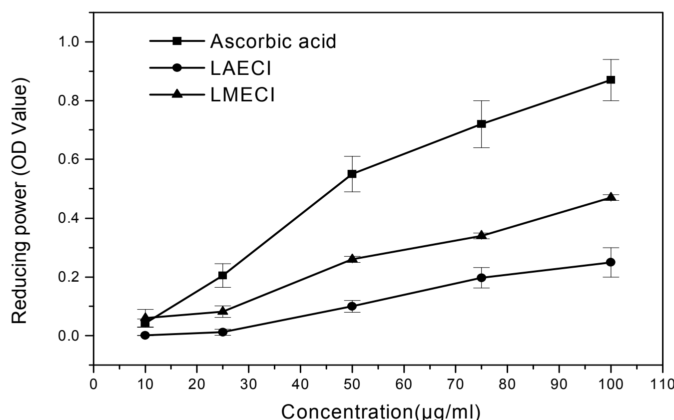

Figure 2
Showing Fe^{3+} reducing power of LMECI and LAECI.

Total Phenolic content

The phenolic (tannic acid equivalent) content was expressed as mg/g of plant dry powder. The total phenolic contents of LAECI and LMECI were respectively 97±9.2 and 140.28± 17.1 mg tannic acid equivalent per gram of dried leaf powder (Fig.3).

Total flavonoid content

Here the total flavonoid content was measured from LAECI and LMECI and expressed as quercetin equivalent. The flavonoid contents of LAECI and LMECI were respectively 88.94±2.94 and 177.06±5.29 mg/g of dried leaf powder (Fig.5).

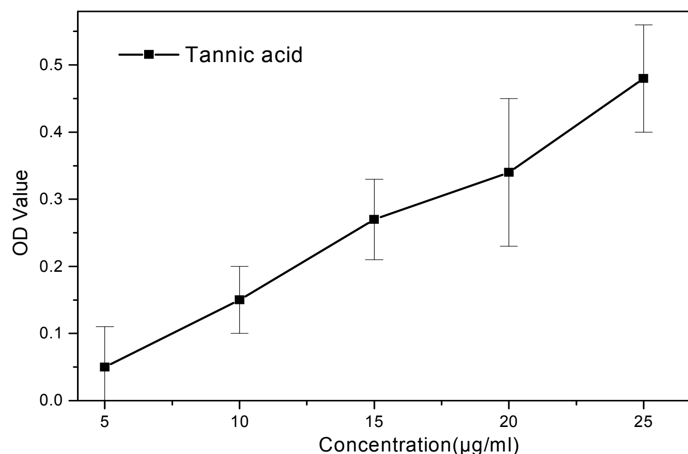

Figure 3
Standard plot of tannic acid.

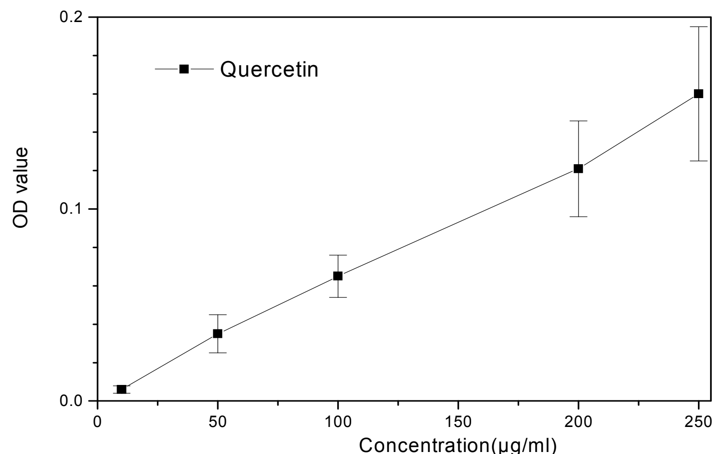

Figure 4
Standard plot of quercetin.

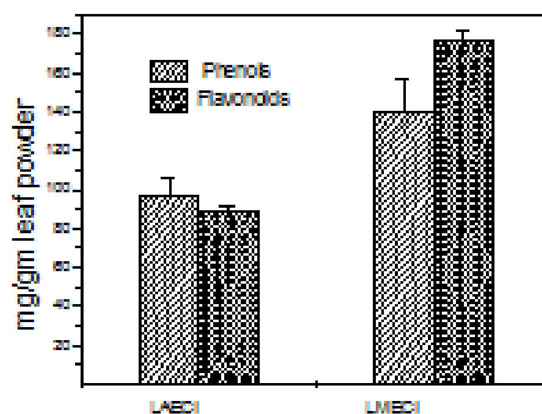

Figure 5
Showing total phenols and flavonoids content in LAECI and LMECI (Values were calculated at the level of dried leaf powder).

Table 1
Different phytochemicals detected in LAECI and LMECI with the various conventional procedures.

S. No.	Phytochemicals	Tests Performed	Results	
			LAECI	LMECI
1	Tannins	$FeCl_3$ test	+	+
2	Saponins	Froth test	-	-
3	Steroids	Kantamreddi et al., 2010	_	-
4	Terpenoids	Kantamreddi et al., 2010	+	+
6	Alkaloids	Mayer's test	+	+
		Wagner's test	+	+
7	Flavonoids	Shinoda test	+	+
		Alkaline solution test	+	+
8	Anthraquinones	Borntrager's test	-	-
9	Phlobatannins	HCl test	+	+
10.	Carbohydrates	Benedict's test	+	+
11.	Glycosides	Alkaline reagent test	+	+

+; present, -; absent.

Preliminary Phytochemical detections
Preliminary Phytochemical analysis revealed that LAECI possesses tannins, more terpinoids, alkaloids, flavonoids, phlobatannins, carbohydrates, glycosides and LMECI possesses tannins, terpinoids, alkaloids, flavonoids, more phlobatannins, carbohydrates and glycosides (Table 1).

DISCUSSION

A number of diseases are related to oxidative stress and free radical-induced damage. Antioxidants are compounds which check the oxidation of organic molecules and thus protect the body from various chronic diseases. The neutraceuticals and cosmetic industries primarily focus on the importance of natural antioxidant products aiming towards the use of such chemicals as potential medicine. Several studies have documented the antioxidant

activities of the phenolic components[11,12]. Free radical scavenging activity is the most important feature of poly phenols and flavonoids. Flavonoids stabilize the free radicals by reacting with the radicals. *C. inophyllum* leaves are frequently used in herbal medicine. Here *in vitro* antioxidant potentials of leaf aqueous (LAECI) and methanolic (LMECI) extracts of *C. inophyllum* were studied in relation to the total phenol and flavonoid contents. The chemical analyses of LAECI and the LMECI showed the presence of tannins, terpenoids, alkaloids and flavonoids (Table 1). The LMECI and LAECI showed effective DPPH free radical scavenging activity starting from very low concentration of extracts and were comparable to ascorbic acid standard. Here, the LMECI has shown more potency in DPPH free radicals scavenging activity than the LAECI and that may be due to the presence of more phenol and flavonoid contents.

A potent antioxidant scavenges ROS and inhibits lipid peroxidation and relatively more antioxidant capacity of a compound may be attributed to the increase in its reducing power. The Fe^{3+} reducing power assay result indicating efficient reducing power, signature for a potent antioxidant, of LMECI and LAECI. The LMECI contained more phenol and flavonoid contents than the LAECI. The leaf aqueous and methanolic extracts of *C. inophyllum* displayed the presence of high amount of phenolic and flavonoid contents. Both of these secondary metabolites have efficient antioxidant activity and their effects are significant on the human health and disease prevention. Plant's polyphenols are credited enormously to their pharmacological activities and there persists strong correlation between high phenolic content and antioxidant activity[13,15]. These results indicate that poly phenols and flavonoids contributed significantly to the antioxidant capacity in LAECI and LMECI. This study also confirms the findings of previous studies showing excellent linear correlations between phenolic contents and antioxidant capacity of phytochemicals [27-29]. In the present study the significant correlations were observed between the total phenol contents and the estimated reducing power and antioxidant activities of LMECI and LAECI. Here LMECI seems more promising for antioxidant potentials than LAEC. In conclusion, it may be said that the leaf aqueous and methanolic extracts of *C. inophyllum* possess an excellent free radical scavenging potential.

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